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Detection of Orally Toxic Microbial Metabolites in Foods with Bioassay Systems[1]

Nachweis von per os toxischen mikrobiellen Metaboliten in Lebensmitteln durch biologische Teste

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Die von anderen Untersuchern während dieser Arbeit festgestellten spezifischen Empfindlichkeiten wurden bestätigt. Einige Protozoen, Fische, Artemia und besonders Daphnia zeigten sich klinisch anwendbaren Konzentrationen von Histamin gegenüber empfindlich; *Locusta migratoria* und zwei Menschenzellenlinien waren für Staphylokokkenenterotoxine, Daphnia und drei Fischarten für sehr niedrige Aflatoxin B1-Konzentrationen empfindlich.

I. Introduction and Scope of the Investigation

Food control agencies and industries might be interested in a rapid and sensitive biological test system for the detection of bacterial and mycotoxins that may be harmful to man and animals when ingested with foods or feeds.

HUTNER et al. (1962, 1964a, b), HULL (1962), EPSTEIN and BURROUGHS (1962), AARONSON (1960, 1962a, b, 1964a, b) and NATHAN et al. (1958, 1962) have shown that Protozoa may be used for the detection of water soluble carcinogens, acetyl choline, γ-aminobutyric acid, hypocholesteremic agents, antihistamines, pteridines, thalidomide, phenothiazine tranquilizers, herbicides and tobacco smoke. Although BERNHEIMER (1963) found no effect on Protozoa of purified α- and β-toxins of *Staph. aureus* and MCINTOSH and DUGGAN (1965) similarly showed that purified and crude enterotoxin B of *Staph. aureus* did not affect Protozoa, introductory research was carried out on the sensitivity of Protozoa to some bacterial and mould toxins that have led to outbreaks of foodborne diseases in man or animals. These included: (I) Aflatoxin, produced mainly by the mould *Aspergillus flavus*; (II) type A enterotoxin of *Staph. aureus*; (III) the types A, B and E of toxins produced by *Clostridium botulinum*; (IV) histamine, formed e.g. by *Proteus morganii*; (V) endotoxins of enteropathogenic strains of *E. coli*.

Because Protozoa appeared probably not to be sensitive to all toxins of microbial origin occurring in foods, the suitability of other test organisms for the detection of toxic substances was also studied. Included were (I) different types of fish as used by RABINOWITZ (1966) for the detection of dimethylsulfoxide and radioactive dyes, by DOWDEN and BENNETT (1965) for the detection of polluants in water and by ABEDI and MCKINLEY (1967, 1968) for the bioassay of captan and mycotoxins; (II) small animals such as Daphnia's and Artemia's which were studied for their suitability for the detection of botulinus toxin and have been suggested by SUEME (1957) for the detection of toxic substances in general; (III) embryonated hen's eggs already used for the detection of endotoxin by OHBA (1959), MCLAUGHLIN (1963) and VERRETT (1964); (IV) larvae of grasshoppers, cockroaches and catterpillars as introduced by DOWDEN and BENNETT (1965) for the detection of polluting chemicals in water; (V) eggs of the cotton bug, suggested for this purpose by Dr. G. B. STAAL (1968); (VI) frog spermatozoa found useful by STOJANOW and NESTOROW (1968) for the rapid detection of Staphyloenterotoxin and bull spermatozoa; (VII) mammalian cell cultures used for toxin detection by KIENITZ and SCHMELTER (1964), GRIGOROVA and DANON (1968) and PERLMAN (1968).

Finally, some microorganisms were examined for their suitability as bioassay system for certain toxins of microbial origin. CHESTER (1967) reemphasized the usefulness of yeasts, BURMEISTER and HESSELTINE (1966) the suitability of bacteria for some specific purposes. The alga *Chlorella pyrenoidosa* was found sensitive to polyethylene glycol by GREENWAY, HILLER and FLOWERS (1968) and to aflatoxin B, by IKAWA, MA, MEEKER and DAVIS (1968, 1969) and PFANDER (1968).

II. Test Procedures

1. Cultivation and study of Protozoa

Axenic cultures of *Tetrahymena pyriformis, Ochromonas danica, O. malhamensis* were obtained from the Culture Collection of Algae and Protozoa (Cambridge), *Crithidia fasciculata* from Dr. NEAL, Welcome Research Laboratories Beckenham (U.K.), *Euglena gracilis* from Dr. GUTTMAN, Natthan, New York (U.S.A.).

The composition of the different media chosen to obtain both maximal growth and a sufficient conductivity for measurements by the Coulter counter for four of the strains used was the following:

Tetrahymena pyriformis

proteose peptone	10 g	resistance 53.8 k Ω
yeast extract, dried	2.5 g	
distilled water	1 l	

Ochromonas spec.

liver infusion, dried (Oxoid)	1 g	resistance 59.5 k Ω
glucose	1 g	
tryptone	1 g	
NaCl	1 g	
distilled water	1 l	

Euglena gracilis

sodium acetate	1 g	resistance 53.8 k Ω
beef extract, paste (Difco)	1 g	
tryptone	2 g	
yeast extract, dried	2 g	
distilled water	1 l	

Crithidia fasciculata

NaCl	4 g	resistance 30.8 k Ω
Na_2HPO_4	2.5 g	
KH_2PO_4	1 g	
tryptone	20 g	
glucose	5 g	
yeast extract, dried	1 g	
distilled water	1 l	

To the basal medium of Crithida 0.05 g haemin, dissolved in a few drops of 1 N NaOH, is added and thereupon the medium is diluted to two liters with distilled water. Finally the pH is adjusted to 7.8–8.0. This medium is called "PHYG medium" (Dr. NEAL, personel information).

All Protozoa were grown under a set of Philips "daylight" lamps no. 29 and no. 55, the former with a peak at 5900 Å, the latter with a band between 4500 and 6700 Å (ELENBAAS, 1959). The incubation was carried out at room temperature (18–22° C) for 1–5 days, depending on the time required for the culture to reach its maximal growth.

To determine the influence of the various toxins on these Protozoa in the first instance their growth was measured with a turbidimeter, because tentative assays had shown that the agar diffusion test only offers possibilities in the case of Euglena, which would have unacceptably limited the assays. The chlorophyll containing

cultures of Euglena and O. *danica* were measured in the wavelength range 525–580 mμ whereas for the other strains the band 655–740 mμ was used.

To test the effect of toxins, first of all series of dilutions were made and added in 2.5–3.0 ml quantities to 2.5–25 ml of suitable Protozoa growth media. Subsequently each tube was inoculated with 0.2 ml of a full grown axenic culture. After suitable incubation, the growth of the Protozoa in the presence of different concentrations of toxin was compared with that of the control culture.

Similarly the numbers of cells per 1 ml incubated culture fluid with and without added toxins were determined with the Coulter counter (KUBITSCHEK, 1958).

2. *Cultivation and study of fish and fish larvae*

Cultivation of *Poecilia reticulata* (= guppies) was performed according to FREY (1959).

One or two days' old fishes were used for the assays. Suitable toxin dilutions were made in aquarium water and the fish supplied subsequently. After 4 days the numbers of survivors were determined for each dilution.

One female and one male specimen of *Macropodus opercularis* (paradise fish) were kept in an aquarium without other specimina, and fed with dry Rosawil feed, or, especially in summertime, with tupifex worms and water fleas. When foam was observed on the surface of the aquarium water, indicating that within a few days the young would appear, this was immediately transferred to another aquarium not containing any fish, to avoid plunder. Within a week after hatching the foamy dispersion of eggs was used in the bioassays. For this purpose adequate dilutions of the toxins were made in 50 ml plugged erlenmeyers containing 25 ml aquarium water. Thereupon ten larvae were added and after four days at room temperature the effect of the toxins assessed. The effect was considered positive when the larvae were swollen, i.e. no longer arrow-shaped and/or sank to the bottom.

For the culture of larvae of the zebrafish *(Brachydanio rerio)* the same procedure was applied. Alternately, a grating of parallel glass rods, supported at both ends by two tubes, was placed on the bottom of the aquarium. Only eggs can pass this grid, not the parent fish. The criteria used in the bioassay were the same as in that of paradise fish.

3. *Cultivation and study of Daphnias (Waterfleas)*

Daphnias belong to Crustaceae, Class of Arthropoda, group of the Branchiopoda. In spring and summertime the animal is found in ditches, pools and ponds in all temperate geographical areas. Cultivation during wintertime was performed in an aquarium-container, with sand on the bottom and some plants floating in the water. The food consisted of lettuce. At regular intervals the water was replaced by distilled water to avoid increasing salt concentrations.

For every assay 10 animals of the same size were collected and used for each dilution. The actual toxin exposition tests were carried out in water, adjusted to the optimal pH for toxin action, viz. 6.6. After 2–4 days' exposition at room temperature the numbers of survivors were determined.

4. *Cultivation and study of Artemias (brine shrimp)*

Artemias also belong to the Arthropoda. Their eggs can be obtained in aquarium-shops.

Hatching of the eggs was achieved by exposure for 2–3 days at room temperature layers of < 4 cm of a fluid medium of the following composition (KUENEN, 1939):

KNO_3	0.2 g	$MgCl_2$	0.2 g
K_2HPO_4	0.2 g	NaCl	58.0 g
Na_2SO_4	0.2 g	distilled water	1000 ml.

Immediately after hatching of the eggs, approximately 100 were exposed to various concentrations of toxins. From the degree of sedimentation observed the amount of killing was estimated and expressed in 0,50 or 100%.

5. Tests with embryonated hen's eggs

Series of 3 × 20 healthy embryonated hen's eggs were inoculated by means of syringe at the side of the air cell, albumen and yolk respectively, using 0.05 ml of each toxin dilution under study (GEBHARDT, 1968). Such injections were performed at 0 day and after 4 days of incubation at 37°C. After incubation for 14 days the numbers of survivors were determined.

6. Cultivation and study of some insects

Dr. G.B. STAAL (Entomological Institute, Agricultural University Wageningen, The Netherlands) kindly furnished different species of insects, together with instructions how to handle them. The insects were all kept at room temperature.

Grasshoppers

Only young specimen of the following two species of locusts were used: *Locusta migratoria-migratorioides,* the small one, and *Schistocerca gregaria,* the large one.

During the experiments the locusts were kept in small boxes covered with gauze. The food consisted of fresh sword-grass.

The toxins were tested by injecting the animals with 0.2 ml of a suitable dilution of the toxin in the second segment from the thorax and in the direction of the thorax. A positive reaction was considered to have been obtained when a grasshopper died within 4 days after the injection.

Cockroach (black beetle)

The species *Gromphradorhina portentosa* was used. During the experiments the insects were fed on bread and water, offered in a cottonwool swab in an open glass tube.

Injections of 0.2 ml were given in the neck muscles under the thorax so that the needle was nearby parallel to the wingcase and in the length axis of the body. The numbers of survivors were again determined after 4 days.

Caterpillars

One of the types of caterpillars used was the blue and white coloured *Philosamia cynthia* which was fed on fresh bird cherry leaves *(Prunus serotina).* The other type was the green coloured *Actias selene* fed on leaves of the rhododendron. The caterpillars were used in the non-metamorphic stage of their life cycle.

Injections of 0.2 ml fluid were given behind the head in the body by stabbing the needle parallel to the body surface in the direction of the tail. Survivors were determined as described above.

7. *Cultivation and study of cotton bug eggs*

Dr. G.B. STAAL (Entomological Institute, Agricultural University Wageningen, The Netherlands) kindly furnished the eggs of the cotton bug *Dysdercus völkeri* and the insects, together with instructions how to handle these.

The eggs and the mature insects were kept at room temperature. The insects were placed in a box with a double layer of gauze, the underlayer of which had a mesh size that prevents the eggs from passing. On the bottom a moistened cotton plug was placed to avoid the drying out of the eggs. The upper gauze layer was provided with a tube with water closed by a cotton plug. The bugs were fed with sunflowers pips.

For testing the activity of toxins, two strips of "Scotch" brand drafting tape no. 230 were fixed to the bottom of a petri dish. With the help of a smooth, wet brush the eggs were taken from the 0–24 h old crop and 20 of them fixed to one strip. Subsequently, using a 1 ml syringe, a drop of 0.07 ml of the solution of the toxins to be tested was brought on the eggs. Before closing the petri dish a wet cotton plug was added to prevent desiccation of the eggs. After 5 or 6 days holding at room temperature the degree of hatching of the eggs was determined.

To increase the adsorption of the toxin, in some assays 0.01 respectively 0.1% undecylenic acid was added to the respective toxin dilutions.

8. *Harvesting and testing of the susceptibility of frog spermatozoa*

The procedure of STOJANOW and NESTOROW (1968) was followed.

Male frogs were caught in ditches and meadows. The optimal condition of the frog for this purpose prevails in the months of May and September. Because the spermatogenesis of frogs in captivity degenerates rapidly, the animals were used on the day when they were caught. They were injected, in their dorsal lymph sac, with 1 ml pregnyl i.e. 100 i.u. gonadotrophinum chorionicum (N.V. Organon, Oss, The Netherlands). After one hour a small pipette was introduced in the cloaca and, if the sperma had not been voided while urinating due to handling, a mixture of urine and sperma was collected in this way.

The sperma so obtained were mixed with suitable toxin dilutions. The effect of the toxins was studied microscopically, the motility of the cells being taken as the criterion. When the motility was lost due to exposure to the toxin the response was considered positive.

9. *Obtaining and study of bull spermatozoa*

Dr. J. HENDRIKS (Institute of Veterinary Obstetrics, unit K.I., State University Utrecht, The Netherlands) kindly furnished fresh bull spermatozoa. Dilutions of these were made in a solution containing 2.9 g sodium citrate, 10 ml egg yolk and 100 ml distilled water.

Adequate dilutions of the toxins were mixed with the egg yolk suspension of spermatozoa in small wells of microscopic slides. After 2–3 hours the effect of the toxins was assessed microscopically, using the motility of the spermatozoa as the criterion. When the control, i.e. the suspension of bull spermatozoa without toxin added, became non-motile, the experiment was terminated.

10. *Preparation and study of tissue cultures*

General

The procedures, recommended by the Virology Department of the National Institute of Public Health at Bilthoven, The Netherlands, were carefully followed.

Special silicone tubing and butyl rubber stops were used to avoid killing of cells. Glassware was cleaned with solution of the detergent 7X, obtained from AVAC N.V. (Naarden, The Netherlands), subsequently rinsed several times with tap water and finally with twice-distilled water. A cornwall syringe, code 1250S, 1250MH, 1220FO, with bent needle, code 1250NR, and luer locking, was used in combination with a closed system consisting of a screw-capped ("transfusion") bottle.

Cell growth in the tubes was studied with the aid of a Zeiss microscope, equipped with objectives under the microscope table.

Cell lines

Human embryonic lung cells

These were obtained from Flow Laboratories, Irvine Ayrshire, Scotland (catalogue no. 0–482 (1968) and brought over by plane.

Aberrant tubes were discarded, the others incubated overnight at 37° C. Before further use, the viability of the cells was assessed by inspecting the cultures microscopically. Every three days, the medium in each tube was replaced by fresh EAGLE medium (cf Media used for tissue cultures.)

In the actual tests the tubes were incubated at 37° C, at a slope of 5°, which ensured that the cell layer was covered by the medium. A toxin preparation was considered effective when the cell layer was interrupted; this effect was normally attained within 5 × 24 hours.

HELA-cells

The strain RIV '58 was obtained from Dr. KUNST, Virology Department College of Veterinary Medicine, Utrecht University.

Aberrant cultures were discarded. Before further use, the viability of the cells was assessed by inspecting the cultures microscopically. It was not found necessary to replace the medium during an experiment requiring at most 6 days as long as the cell layer was well covered by the medium.

A toxin preparation was considered effective, when the cell layer was interrupted. Such an effect was mostly attained within 5 × 24 hours.

Mice fibrinoblast cells

Mice muscle fibrinoblasts were also obtained from Dr. Kunst.

Rhesus monkey kidney cells

Rhesus monkey kidney cells were purchased from Flow Laboratories, Irvine Ayrshire, Scotland (catalogue no. o-332 (1968) and forwarded by plane.

Aberrant cultures were discarded, regular ones incubated during one night at 37° C.

Before further use, the viability of the cells was assessed by inspecting the cultures microscopically. Every three days, the medium in each tube was replaced by fresh (medium for monkey kidney cells).

In the actual tests the tubes were incubated at 37° C at a slope of 5° which ensured that the cell layer was covered by the medium. A toxin preparation was considered effective when the cell layer was interrupted. Such an effect was, as a rule, attained within 5 × 24 hours.

Media used for tissue cultures

Medium for human lung cells

Every three days all tubes with cells were resuscitated by the addition ot the following fresh, sterile medium:

MEM = minimum essential medium, according to EAGLE (1959), with HANK's salts, cat. no. 1-022E	100 ml
inactivated (30 min/56°C) calf serum, cat. no. 4-020D	3 ml
glutamine (2.92 g in 100 ml aqua bidest)	1 ml
sodium bicarbonate 1.4%	7.5 ml
penicillin (100 E/ml)/streptomycin (100 μg/ml) solution	0.4 ml

Medium for HELA-cells

Quantities of 1 ml per tube of the following medium were used:

T.C.-medium 199 (10 × concentrated) Difco 5696-72	5 ml
inactivated (30 min/56°C) calf serum	6 ml
lactalbumin hydrolysate (NBC) 2.5% solution	12 ml
solution of antibiotics[3]	0.2 ml
sigma solution[4]	2.5 ml
distilled water	39 ml

Medium for fibrinoblast cells

Quantities of 1 ml per tube of the following medium were used:

Gey C	2 ml
Inactivated calf serum	5 ml
HANK's solution	5 ml
lactalbumin hydrolysate 2.5% solution	10 ml
penicillin (100 E/ml)/streptomycin (100 μg/ml) solution	0.3 ml
distilled water	28 ml

The formulae of the component solutions were:

1. Gey C-solution:

$NaHCO_3$, Merck 6329	2.25 g
distilled water	100 ml

CO_2-gas is bubbled through, until pH 7.9 is reached.

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[3] This solution has the following composition:

10^6 U penicillin G-Na
1 g streptomycin
5×10^5 U nystatin
5×10^5 U polymyxine B sulphate
(= 50 mg polymyxine B standard)
20 mg folin acid (USP XVII)
40 ml physiological saline

[4] This solution consists of:

2.4 g tris (= hydroxy methyl amino methanum (Tham))
10 mg phenol red (Merck 7241)
100 ml distilled water.

Sterilisation by filtration.

When kept in the refrigerator at 4°C this solution is stable during almost 3 months.

2. HANK's medium:

NaCl	80	g
KCl	4	g
KH_2PO_4	0.6	g
glucose	10	g
phenol red	0.2	g
$NaHCO_3$	3.5	g
$CaCl_2$ (*)	46.6	g
$MgSO_4$ (*)	2.05	g
$MgCl_2$ (*)	2.13	g
Na_2HPO_4 (*)	0.75	g
distilled water	100	ml

The salts marked with a (*) have to be added one after the other, subsequent to dissolution of the other ingredients listed.

Medium for monkey kidney cells

Melnich's B medium was used for cultivating monkey kidney cells. It has the following composition:

HANK's medium (cf Medium for fibrinoblast cells)	1000 ml
lactalbumin hydrolysate	5 g
inactivated (30 min/56°C) calf serum	20 ml

Diluents

Dilutions of toxins, used in tissue culture assays, were made with the so-called P.B.S. solution according to DULBECCO and VOGT (1954). This diluent consists of three parts:

Component A –	NaCl	8.0 g
	KCl	0.2 g
	$Na_2HPO_4.2H_2O$	1.15 g
	KH_2PO_4	0.2 g
	phenol red 0.2%	10 ml
	aqua bidist.	800 ml
		sterilized 10 min/120°C
Component B –	$CaCl_2$ anhydr.	0.1 g
	aqua bidist.	100 ml
		sterilized 10 min/120°C
Component C –	$MgCl_2.6H_2O$	0.1 g
	aqua bidist.	100 ml
		sterilized 10 min/120°C

After cooling to room temperature, components A, B and C were mixed in the quantities indicated.

11. Cultivation and study of yeasts

Cultivation of the two species studied, viz. *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae,* was performed in a 2% glucose/0.5% yeast extract broth of pH = approximately 6.6 (MOSSEL, 1962). Suitable toxin dilutions were made in this medium, which were then added at the beginning of the logarithmic growth phase of the yeast culture, where the cells are most sensitive. Incubation was carried out at 30° C.

The effect of the toxin was assessed turbidimetrically at λ = 655–740 mμ by comparing the course of the growth of the control culture without toxin and in the presence of varying toxin concentrations.

12. Use of bacteria

Three types of test bacteria were used viz. aerobic Gram-positive ones *(Bacillus cereus, Bac. pumilus* and *Bac. subtilis),* an anaerobic Gram-positive rod *(Cl. sporogenes)* and a Gram-negative type *(E. coli).*

Suitable dilutions of various toxins were made in 10 ml tubes containing sterile brain heart infusion broth. These were then inoculated with approximately 0.05 ml of freshly prepared cultures of the test organisms and incubated overnight at 30° C; in the case of the anaerobic bacterium under a seal of sterile beef tallow.

The effect of the presence of the toxins was estimated turbidimetrically at λ = 655 –740 mμ.

13. Use of Chlorella vulgaris and Chlorella pyrenoidosa

Cultivation was performed under Philips daylight lamps, colour no. 55 and 29, at room temperature. RODHE's liquid medium, having the following composition, was used for culturing in this instance.

$MgSO_4 \cdot 7H_2O$	100 mg
KH_2PO_4	100 mg
NH_4NO_3	600 mg
KNO_3	300 mg
$NaHCO_3$	700 mg
$Ca(NO_3)_2$	100 mg
Fe. EDTA[5]	50 ml
Trace elements solution[6]	20 ml
Distilled water	10000 ml

Suitable toxin dilutions were made in this medium. The effect of the toxin was assessed by comparing, turbidimetrically at λ = 525–580 mμ, the course of the growth of the control culture without toxin with the culture obtained in the presence of varying toxin concentrations. The incubation temperature was 20° C.

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[5] Fe. EDTA consists of: 800 mg EDTA ($C_{10}H_{16}N_2O_8$) = Titriplex II
1000 mg $Fe_3(NH_4)_2SO_4 \cdot 24H_2O$.
in 1000 ml distilled water.

[6] The trace elements solution consists of:

$Na_2B_4O_7 \cdot 10H_2O$	2 g
$MnSO_4 \cdot 4H_2O$	0.3 g
$ZnSO_4$	0.2 g
$(NH_4)_6Mo \cdot O_{24} \cdot 4H_2O$	0.02 g
$CuSO_4 \cdot 5H_2O$	0.5 g
$Al_2(SO_4)_3$	0.2 g
$Co(NO_3)_2 \cdot 6H_2O$	0.5 g
EDTA	4.3 g
distilled water	1000 ml

In addition, growth tests on solid media were carried out. A fresh culture of *Chlorella vulgaris* or *Chl. pyrenoidosa* was flooded over plates of Chlorella-agar (Difco, 1966). After drying, glass cups respectively paper discs were mounted. The glass cups were filled with suitable toxin dilutions. The paper discs were impregnated with the same toxin dilutions. The effects of the toxins were studied after 5–6 days incubation at 20° C, by measuring the inhibition zones, if any, around the cups and discs respectively.

III. Production or Procurement of Toxins

1. General

It was decided to produce most of the toxins at the Central Institute for Nutrition and Food Research TNO. The reasons were twofold. First, the majority of these toxins appeared not to be commercially available and could not be obtained from other laboratories engaged in this field. No impression of the effects of possibly concomitantly present impurities in the test systems could be gained either when only pure toxins prepared elsewhere were used.

2. Aflatoxin

The production of aflatoxin was performed in cotton plugged 5 l penicillin bottles. Three different strains of the mould *Aspergillus flavus* were used, viz. strain ATCC 15517, which mainly forms component B of the aflatoxin complex, strain M 93 C z Dox, which mainly forms component G and strain CB 5 Link van Beyma, which forms neither B nor G toxin. Each bottle contained 150 ml of the synthetic medium suggested by ADYE and MATELES (1964) and circa 30 g unroasted ground peanuts, previously extracted with ether in a Soxhlett-apparatus. After inoculation with the mould, incubation was carried out during a fortnight at 26° C. Extraction of the crude toxin was performed with $CHCl_3$ with 1% ethanol added as described by MATELES and ADYE (1965).

This extract was purified by thin layer chromatography, using Kieselgel G, and also by column chromatography. For the preparation of the purified components B and G of the aflatoxin complex the set of aids for isolation in chromatography according to Stahl, as manufactured by DESAGA, Heidelberg, Germany (cf. Catalogue of this firm) was used.

After this process of purification the degree of purity of the toxin components was determined with an infrared spectrophotometer, comparing the spectra obtained with those published by HARTLEY et al. (1963). The concentration of the toxin component in the preparation was then estimated by comparing it with the reference substance obtained from Ir. J.A. VAN DER LINDE (National Institute of Public Health, Bilthoven, The Netherlands). In this way a toxin potency of 150 μg/ml of the solvent used, i.e. chloroform with 1% ethanol, was obtained ultimately.

For testing the various biological effects of the aflatoxin components B and G, an equal volume of 0.5% tween 80 in sterile water was added to the alcoholic chloroform solutions of the toxins and the organic solvents evaporated subsequently. The same procedure was always applied to the controls used in the same biological test system.

3. Botulinum toxins

Production and testing of botulinus toxin type E

Clostridium botulinum, strain 1537/62 (ROBERTS, 1965), was used for the production of type E toxin. The procedure of SAKAGUCHI et al. (1961, 1964) was followed, with the exception that a medium of the following composition was used:

glucose	10	g
trypticase	15	g
yeast extract	5	g
cysteine HCL	0.75	g
distilled water	1000	ml

pH adjusted to 7.2.

The preparations were assayed by injecting six mice of 18 g weight intraperitoneally with 0.5 ml of a series of dilutions. The diluent was composed as follows:

gelatin	2 g
0.2 M Na_2HPO_4	200 ml
0.2 M KH_2PO_4	300 ml
distilled water	500 ml

pH adjusted to 6.5.

A positive reaction was considered to have been obtained, when a mouse died within 4 days after the injection and under obvious symptoms of botulism.

In this way a final preparation with a potency of circa 10^6 MLD/ml was obtained. In susceptibility tests made with this preparation, toxin in a concentration 10^2–10^4 MLD was used and the pH was adjusted to 6.6 in order to reach an optimal toxic effect. At the completion of each test, the residual toxin level in the medium was assayed by means of the method described above. As a control, a preparation containing 10^4 MLD/ml toxin, heated during 5 minutes at 100°C was applied to the same biological assay system.

Production and testing of botulinum toxins types A and B

A strain of *Clostridium botulinum* received from Dr. BOROFF (USA) was used for the production of the type B toxin. It was produced and purified according to BOROFF (1955), with the exception that N-Z amino acids (Cheffield Chemical, Norwick New York) and cysteine HCl were used in the culture medium. The final preparation obtained had a potency of 10^7 MLD/ml.

A strain of *Clostridium botulinum,* type A, also received from Dr. BOROFF (USA) was used, and the toxin obtained purified in exactly the same way. The final preparation had a potency of 10^6 MLD/ml.

4. Endotoxins of E. coli

Purified polysaccharides of the *E. coli* toxin types O 111: B_4 and O 55: B 5 were purchased from Difco Laboratories, Detroit, Michigan, USA. The endotoxins were dissolved in physiological saline. The concentrations tested were in the range of 1000 μg/ml to 10 μg/ml.

5. Histamine

Histamine phosphate

This ingredient was obtained from the pharmaceutical trade in a grade, meeting the requirements of the British Pharmacopoeia, Ed. 1958. Dilutions of histamine phosphate were made in the respective media. The final concentrations tested were 1, 0.1, 0.01 and 0.001%.

Production of histamine from histidine by various types of bacteria of common occurrence in foods

Twenty-four strains of common food bacteria were cultivated in yeast extract glucose/L-histidine broth. After 48 hours incubation at 30° C, the culture fluids were examined chromatographically for histamine, using the electrophoretic technique at 300 Volt in a pyridin-glacial acetic acid buffer at pH = 5.6, followed by development of the strips with Pauly's reagents.

A maximum production of 1300 μg histamine per ml (= 0.13%) was obtained, with a strain of *Proteus morganii* 874 (cf. Table 1).

Table 1. Production of histamine by different strains of bacteria upon growth in a histidine containing medium

	histamine (μg per 0.1 ml)
Enterobacter aerogenes	< 0.1
Escherichia coli Vught	< 0.1
Klebsiella 377	< 0.1
Proteus mirabilis	< 0.1
Pseudomonas aeruginosa (36)	< 0.1
Salmonella suis pestifer	< 0.1
Staphylococcus aureus 502	< 0.1
Shigella boydii	< 1
Escherichia coli E.B./F/16/32	< 5
Shigella sonnei	< 5
Clostridium perfringens D 483	5
Clostridium perfringens D 692	5
Escherichia coli	< 10
Salmonella paratyphi A	< 10
Salmonella paratyphi B	< 10
Salmonella enteriditis	10
Clostridium perfringens 4133	50
Proteus morganii 469	100
Proteus morganii	> 100
Proteus morganii 578	> 100
Proteus morganii 627	> 100
Proteus morganii 874	> 100

6. Staphyloenterotoxins

Staphylococcus aureus, strains 196-E and S-6 (CASMAN, 1958, CASMAN et al., 1963) were used for the production of enterotoxin A and B. As an enterotoxin-negative control *Staphylococcus aureus,* strain Wood 46, was used.

The enterotoxin was produced in two different ways. In the one according to CASMAN and BENNETT (1963), toxin production was performed in a cellophane sac contained in a penicillin flask. In the other procedure enterotoxin was produced in screw-capped bottles under a 10% CO_2 atmosphere. After incubation, centrifugating and filtering the supernatant through a sintered glass filter, the filtrate was heated for half an hour at 100° C to destroy all toxins, except enterotoxins. The toxin was then purified by $(NH_4)_2SO_4$-precipitation and dialyzed against phosphate buffer (KATO et al., 1966; CHU et al., 1966). The culture filtrate of strain Wood 46 was treated in the same way.

The concentration of the toxins formed was determined in the kitten test (DOLMAN and WILSON, 1940), which, although rather inaccurate, was the only one which could be conveniently used in the screening tests. If e.g. a 10^2 dilution of a toxin preparation was the highest to give rise to a positive kitten test, the toxin was said to have an effective dosis (= ED) of 100.

The second procedure for the production of enterotoxin, mentioned above, generally yielded higher enterotoxin concentrations.

In addition, a purified preparation of enterotoxin A obtained from Dr. E. SCHANTZ, Fort Detrick (USA) through the intermediary of Colonel J.L. Sullivan, Jr., Army Attache Office The Hague, The Netherlands, was used in the investigations. This preparation was also titrated by intraperitoneal injection of series of decimal dilutions into kittens.

IV. Results and Discussion

All results obtained when the toxins, specified in the section "Production or procurement of toxins", were assayed in the biological test systems and under the conditions described in detail in the section "Test procedures", have been summarized in Table 2. In view of the many very high levels of detection listed, it is obvious that the gross results were rather negative. This is all the more so, because the few positive results obtained pertain to concentrations which, with an occasional exception, are far above the levels of detection attained in methods that are customarily used for the specific assay of a given toxin.

Nevertheless it could be established that the following test systems were sensitive to the chemically more or less significant concentrations of the toxins listed behind their names:

- *Euglena gracilis, Ochromonas danica* and *O. malhamensis:* 0.10% histamine;
- Human lung and *HELA*-cells: 0.5–2 ED staphyloenterotoxins A + B; 500 µg/ml Endotoxin B5;
- *Brachydanio rerio, Macropodus opercularis* and *Poecilia reticulata:* 0.15–0.20 µg/ml aflatoxin B and G; the first in addition: 0.1% histamine; the latter in addition: 10 MLD/ml botulinum type E toxin and 0.10% histamine;
- *Artemia:* 0.10% histamine; 1.5 µg/ml aflatoxin B and G;
- *Daphnia:* 0.015 µg/ml aflatoxin B and G and 0.010% histamine;
- *Locusta migratoria:* 20 ED staphyloenterotoxin A; 0.14% biologically produced histamine;
- *Schistocerca gregoria:* 0.14% biologically produced histamine;
- *Bacillus* and *Clostridium* sspp: 15–40 µg/ml aflatoxin B and G;
- *Chlorella pyrenoidosa:* 0.2% histamine.

Table 2. Summary of all results obtained

Assay systems	Detectable level of toxins in units indicated										
	Aflatoxins (μg/ml)		Botulinum toxins (MLD/ml)			Endotoxins (μg/ml)		Histamine (%)		Staphylo-enterotoxins (ED)	
	B	G	A	B	E	B_4	B_5	C	B	A	B
Crithidia fasciculata	> 1.5	> 1.5	$> 10^3$	$> 10^3$	$> 10^2$	> 20	> 20	> 1	> 0.07	> 10	> 10
Euglena gracilis	> 1.5	> 1.5	$> 10^4$	$> 10^2$	$> 10^2$	> 20	> 20	0.1*	> 0.07	> 10	> 10
Ochromonas danica	> 1.5	> 1.5	$> 10^3$	$> 10^3$	$> 10^2$	> 20	> 20	0.1*	0.07	> 10	> 10
O. malhamensis	> 1.5	> 1.5	$> 10^3$	$> 10^3$	$> 10^2$	> 20	> 20	0.1*	> 0.07	> 10	> 10
Paramecium aurelia	N.T.	N.T.	$> 10^3$	$> 10^3$	$> 10^3$	> 20	> 20	N.T.	> 0.07	N.T.	N.T.
Tetrahymena pyriformis	N.T.	N.T.	$> 10^3$	$> 10^2$	$> 10^3$	> 20	> 20	> 1	> 0.07	> 10	> 10
Brachydanio rerio	0.20*	N.T.	$> 10^2$	$> 10^2$	$> 10^2$	$> 10^2$	$> 10^2$	0.1*	N.T.	N.T.	> 10
Ciphophoris helleri	N.T.	N.T.	10^3	$> 10^3$	$> 10^3$	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Macropodus opercularis	0.20*	N.T.	10^3	10^3	10^3	$> 10^2$	$> 10^2$	> 1	N.T.	> 1	> 10
Poecilia reticulata	0.15*	0.15*	$> 10^3$	$> 10^4$	10	$> 10^3$	$> 10^3$	0.1*	N.T.	N.T.	> 1
Artemia	1.5	1.5	$> 10^3$	$> 10^3$	$> 10^3$	$> 10^3$	$> 10^3$	0.1*	N.T.	N.T.	> 10
Daphnia	0.015*	0.015*	$> 10^3$	$> 10^3$	$> 10^3$	$> 10^3$	$> 10^3$	0.01*	N.T.	N.T.	> 10
Hen's eggs, embryonated	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	> 0.1	N.T.	> 2	> 2
Dysdercus völkeri	> 10	N.T.	$> 10^4$	$> 10^5$	$> 10^4$	$> 10^3$	$> 10^3$	> 1	> 0.14	> 150	> 10
Gromphradorhina portentosa	$> 10^2$	N.T.	$> 10^3$	$> 10^4$	$> 10^4$	$> 10^3$	$> 10^3$	> 1	> 0.14	$> 10^2$	> 5
Locusta migratoria	> 0.1	N.T.	$> 10^3$	10^4	$> 10^4$	$> 10^3$	$> 10^3$	> 1	0.14*	20	> 5
Philosamia cynthia	> 20	N.T.	$> 10^3$	$> 10^4$	$> 10^4$	$> 10^2$	$> 10^3$	> 1	> 0.14	$> 10^2$	> 10
Schistocerca gregoria	> 0.1	N.T.	$> 10^3$	10^4	$> 10^4$	$> 10^3$	$> 10^3$	> 1	0.14*	10^2	> 5

Table 2. Continued

Assay systems	Detectable level of toxins in units indicated										
	Aflatoxins (μg/ml)		Botulinum toxins (MLD/ml)			Endotoxins (μg/ml)		Histamine (%)		Staphylo-enterotoxins (ED)	
	B	G	A	B	E	B_4	B_5	C	B	A	B
Bull spermatozoa	> 20	N.T.	> 10^3	> 10^3	> 10^3	> 10^2	> 10^2	> 0.1	> 0.14	> 10	> 10
HELA-cells (human)	> 20	N.T.	N.T.	N.T.	N.T.	> 10^3	500*	> 1	> 0.07	1*	2*
Kidney cells (monkey)	> 20	N.T.	> 50	> 50	> 50	> 10^3	10^3	> 1	> 0.07	> 1	> 2
Lung cells (human)	> 20	N.T.	N.T.	> 10^3	> 10^3	> 10^3	500*	> 1	> 0.14	1*	0.5*
Muscle fibrinoblasts (mouse)	> 20	N.T.	N.T.	N.T.	N.T.	> 10^3	10^3	> 1	> 0.07	N.T.	> 4
Saccharomyces carlsbergensis	> 20	N.T.	> 10^5	> 10^5	> 10^5	> 10^2	> 10^2	> 1	> 0.07	> 15	> 1
Saccharomyces cerevisiae	> 20	N.T.	> 10^5	> 10^5	> 10^5	> 10^2	> 10^2	> 1	> 0.07	> 15	> 1
Bacillus cereus	15	40	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Bac. pumilus	15	20	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Bac. subtilis	15	40	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	> 10
Clostr. sporogenes	15	40	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Esch. coli	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	> 10
Chlorella pyrenoidosa	> 0.1	N.T.	> 10^3	> 10^3	> 10^3	> 10^2	> 10^2	0.2	> 0.14	> 1	N.T.
Chl. vulgaris	> 10	> 10	> 10^2	> 10^4	> 10	> 10^2	> 10^2	> 0.1	> 0.14	> 150	> 10
Number of organisms, per 32 total, sensitive	9	7	2	4	2	0	4	8	3	4	2

N.T. = not tested
* = clinically interesting

The overall conclusions of this investigation are in agreement with many reports in the literature. No *universal* bioassay system is known, so far, that enables the detection of all, or at least a considerable proportion of, toxins of microbial origin that cause disease in humans when absorbed with food. Hence, the situation here is essentially different from the possibilities of detection of almost all antimicrobial food preservatives, attainable when a suitable set of test organisms is chosen. Food investigation laboratories will, therefore, have to use a set of different bioassays for the detection and quantification of microbial toxins of oral pathogenicity in foods and feeds.

The results obtained in this study confirm the usefulness of fish and some bacilli for the detection of aflatoxin (BAUER and LEISTNER, 1969) while the high sensitivity of Daphnia to aflatoxins is quite promising. One fish species, *Poecilia reticulata* is the only test animal in this study that is sensitive to botulinum toxin type E (BORKER et al. 1966). In addition we have established the suitability of some Protozoa, some fish, *Chlorella pyrenoidosa,* Artemia and particularly Daphnia for the assay of histamine. Most test systems studied with the exception of embryonic human lung cell lines and HELA-cells appeared refractory for the staphyloenterotoxins as observed for kidney and fibrinoblast tissue cultures by GRIGOROVA and DANON (1968). However, *Locusta migratoria* was found somewhat sensitive to staphyloenterotoxin A. This observation merits further study, particularly with regard to the reactions of this grasshopper towards the other staphyloenterotoxins, which were not, or only in insufficient quantities, available for study in The Netherlands.

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